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CYTOCHROME P450 SPECIFICITIES OF ALKOXYRESORUFIN O-DEALKYLATION IN HUMAN AND RAT LIVER

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Abstract—The O-dealkylations of ethoxyresorufin and pentoxyresorufin are widely used activity probes for measuring the cytochrome P450 forms, CYP1A1 and CYP2B1, respectively, and their induction by xenobiotics, but there is confusion in the literature about which P450 forms are detected in human and rat liver microsomes by these and homologous alkoxyresorufins. High performance liquid chromatographic analysis confirmed that O-dealkylation to resorufin was the sole or predominant route of metabolism for both short-chain and long-chain alkoxyresorufins and benzyloxyresorufin by rat liver microsomes. The purified 3-methylcholanthrene (3MC)-induced rat P450 forms, CYP1A1 and CYP1A2, and a possible variant form, CYP1A1*, showed substrate selectivities for propoxyresorufin, methoxyresorufin and ethoxyresorufin, respectively. Purified phenobarbitone (PB)-induced CYP2B1 was selective for benzyloxyresorufin and pentoxyresorufin. Purified constitutive CYP2C6 was much less active than CYP2B1 or the CYP1A forms but showed distinctive selectivity for benzyloxy-, propoxy- and butoxyresorufin. CYP1A2 and CYP2C6 metabolised *n*-propoxy- and *n*-butoxyresorufin much more rapidly (8–23-fold) than iso-propoxy- and iso-butoxyresorufin, whereas CYP1A1 and CYP2B1 showed only small differences (2–5-fold) between the *n*- and *iso*-homologues and CYP1A1* and CYP2B2 did not discriminate between them. The results show that ratios between different alkoxyresorufin O-dealkylation (AROD) activities can be more useful than absolute values of single activities for identifying P450 forms. Anti-P450 antibody and furafylline inhibition of rat liver microsomal AROD confirmed that ethoxyresorufin was a selective probe for CYP1A1 in 3MC-induced and isosafrole (ISF)-induced microsomes and that pentoxy- and benzyloxyresorufins both selectively measured CYP2B1 in PB-induced and ISF-induced microsomes. Ethoxyresorufin was *not* a selective probe for CYP1A in liver microsomes from untreated or PB-induced rats, however, where it was metabolised mainly by CYP2C6 and CYP2B1, respectively. Pentoxyresorufin and benzyloxyresorufin were metabolised by several different P450 forms in non-induced rat liver microsomes but mainly by the CYP1A subfamily in 3MC-induced microsomes and by CYP2B1 in PB- and ISF-induced microsomes. Although with purified rat P450s methoxyresorufin appeared not effectively to discriminate CYP1A2 from CYP1A1, CYP1A1* or CYP2C6, furafylline inhibition indicated that methoxyresorufin was a selective measure of CYP1A2 in uninduced and 3MC-induced rat liver microsomes but not in ISF- or PB-induced microsomes. In human liver microsomes, antibody inhibition and furafylline inhibition showed that ethoxyresorufin and methoxyresorufin were metabolised mainly by CYP1A2, whilst benzyloxyresorufin metabolism was due mainly to the CYP3A subfamily but also involved CYP1A2 and CYP2A6. There was considerable interindividual variation in the roles of different P450 forms in all three reactions in human liver.

Key words: metabolism; microsomes; antibodies; induction; inhibition; furafylline

Ethoxyresorufin was originally synthesised as a fluorigenic cytochrome P450 substrate with the aim that the long-wavelength fluorescence of the expected *O*-desmethyl metabolite, resorufin, would allow metabolite formation in microsomal suspensions to be measured by direct fluorimetry without interference from microsomal turbidity or the (blue) fluorescence of NADPH [1]. EROD‖ was found to be a highly sensitive and unexpectedly selective measure of the induction of the P450 form, P448 (CYP1A1), by polycyclic aromatic hydrocarbons in rats [2, 3], whilst the subsequent synthesis of a homologous series of alkoxyresorufins [4] led to the discovery that PROD was a highly sensitive and selective measure of the induction of a different P450 form, CYP2B1, by PB [3]. EROD and PROD are now probably the most commonly used assays for CYP1A1 and CYP2B1, respectively. More recently, BROD and MROD have also begun to be widely used: BROD as a relatively non-selective probe in rats for measuring P450 induction by polycyclic aromatic hydrocarbons (i.e. CYP1A

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‖ Abbreviations: Ab, antibody; ANF, alpha-naphthoflavone; AROD, alkoxyresorufin O-dealkylation; BROD, benzyloxyresorufin O-dealkylation; DLPC, dilauroylphosphatidyl choline; EROD, ethoxyresorufin O-deethylation; ISF, isosafrole; 3MC, 3-methylcholanthrene; MROD, methoxyresorufin O-demethylation; PB, phenobarbitone; PROD, pentoxyresorufin O-dealkylation; PrROD, *n*-propoxyresorufin O-dealkylation.

induction), PB (i.e. CYP2B1 induction) or steroids (i.e. CYP3A induction) [3, 5], and MROD as a selective probe for CYP1A2 [6, 7] on the basis that the ratio between MROD and EROD activities is high for CYP1A2 but low for CYP1A1 [8, 9]. EROD, BROD and MROD are also increasingly being used to measure and characterise P450 activity in human tissues *in vitro* [10–17]. More than 1000 research papers published in the period 1975–1993 included the use of one or more of the AROD assays for P450. We have become aware of some ambiguities and errors in the interpretation of these assays, particularly in the question of precisely which form or forms of P450 are detected by the AROD tests in liver microsomes from variously treated or untreated rats or humans. There is, for example, a common assumption that EROD always reflects primarily the activity of CYP1A1, not just in 3MC-induced rat liver but also in control and even PB-induced rat liver. In this report, in order to clarify some of these questions, we have compared the specificities of several different forms of purified rat liver P450 for a wide range of alkoxyresorufin homologues, and have measured the contributions of different P450 forms to the EROD, PROD, BROD and MROD activities of liver microsomes from control and induced rats and from humans. We have also addressed the question of whether resorufin formation is the main route of metabolism for both short-chain and long-chain alkoxyresorufins and for benzyloxyresorufin.

MATERIALS AND METHODS

Alkoxyresorufins were synthesised as described previously [4]. Resorufin was purchased from Molecular Probes, Inc. (4849 Pitchford Avenue, Eugene, OR 97402-9144, U.S.A.), who can also supply the alkoxyresorufins. Furafylline was supplied by Ultrafine Chemicals (Manchester Science Park, Manchester M15 6SY, U.K.). All other chemicals were of analytical grade or equivalent and were purchased from the usual suppliers, as detailed elsewhere [3].

Animal treatments and preparation of P450s, microsomes and antibodies. Adult, male Sprague–Dawley rats were used throughout, bred in either Aberdeen or Edinburgh University (U.K.). Cytochromes P450 were induced by the administration of drugs to rats *in vivo*, and liver microsomes were prepared, as described elsewhere [3]. Various forms of P450 were purified from rat liver microsomes and identified by N-terminal amino acid sequencing as described previously [18] (CYP3A1/2, purified from PB-treated rats, is so named to indicate that it was not possible to differentiate, by either N-terminal sequencing or antibody recognition, between CYP3A1 and CYP3A2). The recently recommended nomenclature for P450 is used [19]. CYP1A1 and CYP1A1* were separate fractions of P450 purified from 3MC-treated rats to specific contents of at least 19 nmol P450/mg protein; they had the same N-terminal amino acid sequence over 23 residues, the same molecular mass on SDS–PAGE (57 kDa) and largely identical elution characteristics during column chromatography, but were resolved during purification on DE52 anion exchange column chromatography, from which they eluted in <10 mM (CYP1A1) and 25 mM (CYP1A1*) potassium phosphate, respectively (data not shown). The purified CYP1A1 and CYP1A1* preparations are listed individually in view of their differing O-dealkylation specificities (see Results), although it is not known whether they are separate forms of CYP1A. Polyclonal antibodies against the purified P450 forms were raised in rabbits as described previously [18].

Human liver samples, obtained with permission from renal transplant donors in the Aberdeen Royal Infirmary within 30 min of circulatory arrest, were stored and used to prepare microsomes as described previously [20]. Clinical histories for the livers are listed in Table 1.

Alkoxyresorufin O-dealkylation. Microsomal AROD activities were routinely measured using our original continuous fluorimetric method [3], with excitation and emission wavelengths set at 530 nm and 585 nm, respectively. For the measurement of furafylline inhibition, up to 80 μL of a 2 mM solution of furafylline in methanol was pre-incubated for 10 min at 37° with the microsomal AROD incubation mixture, including NADPH but lacking substrate, then the substrate was added to start the reaction. Equivalent volumes of methanol solvent alone caused <10% inhibition, which was corrected for in the calculations. The following modified AROD method was used with purified P450: a 50 μg quantity of dilauroylphosphatidyl choline (DLPC, 10 μL of a 5 mg/mL solution in chloroform) was dried on to the walls of a glass test tube by evaporating the solvent with the warmth of the hand. Purified P450 (either 0.01 nmol or 0.1 nmol) and purified NADPH-P450 reductase (7.7 units per nmol P450; 1 unit = 1 μmol cytochrome c reduced/min) were added to the tube, plus 100 mM Tris–HCl buffer, pH 7.6, to a total volume of 50 μL. The lower amount of P450 was used where reaction rates were greater than 5.0 nmol/min/nmol P450. The tube was very gently vortex-mixed, with occasional warming in the hand, until the spot of DLPC was seen to have disappeared, then the components of the monooxygenase system were allowed to associate by standing the mixture for 5 min at 25°. Alkoxyresorufin (5 μM: 10 μL of a 1 mM solution in DMSO) and further Tris buffer (prewarmed to 37°) to a total volume of 2 mL were added, the mixture was poured into a fluorimeter cuvette, which was then placed in the fluorimeter in a thermostatted cell holder at 37°, and the reaction was started by the addition of 250 μM NADPH (10 μL of a 50 mM solution in water). After a suitable period of linear reaction (usually between 0.5 and 5.0 min), the fluorescence was calibrated by the addition of 10 μL of standard resorufin (either 0.01 mM or 0.25 mM solution in DMSO) to the cuvette.

Antibody inhibition. The specificity of recognition by each antibody of the appropriate subfamilies of rat P450, and the orthologous human P450 forms, was assessed by immunoblotting each antibody against each of the purified rat P450 forms studied here, and against rat (both untreated and induced) and human liver microsomes, as described previously

Table 1. Clinical histories for human liver samples

ID	Sex	Age	Drugs	Diseases/ cause of death	Smoking*	Alcohol†
6	M	51	n	hc/a	maj	maj
7	M	33	‡CPZ,PB,PHT,VP	e/sh	min	min
10	M	19	nk	n/a	min	min
13	F	49	‡AMT,CPZ,TFP,VP	n/a	nk	nk
20	F	62	PV	n/a	min	min
21	F	48	nk	cvd/sh	min	min
25	F	41	‡BM,PHT,PN,SB,TP	e,a/a	min	min
26	F	55	‡PHT	e/a	min	min
28	M	40	‡CP	kd/ih	min	min
29	F	63	n	n/ih	nk	min
30	F	63	BH	as/sh	maj	min
31	F	44	CZ	nk/ih	maj	min
38	F	53	‡AT	n/sh	min	min
55	M	60	n	n/ih	min	min

* Smoking is classified as either min (0 or ≤5 cigarettes/day) or maj (>5 cigarettes/day). None of the individuals was exclusively a cigar or pipe smoker.

† Alcohol consumption is classified as either min (nil, social or occasional drinker) or maj (regular or heavy drinker).

‡ Long-term drug treatment (>3 months).

Abbreviations: a, asthma; ac, accident; as, atherosclerosis; AMT, amitryptaline; AT, atenolol; BH, betahistine; BM, beclomethasone; CP, cyclopenthiazide; CPZ, chlorpromazine; cvd, cardiovascular disease; CZ, clorazepate; e, epilepsy; hc, hepatic cirrhosis; ID, individual number; ih, intracerebral haemorrhage; kd, kidney disease; n, none; nk, not known; PB, phenobarbitone; PHT, phenytoin; PN, prednisolone; PV, papaveratrum; SB, salbutamol; sh, subarachnoid haemorrhage; TFP, trifluoperazine; TP, theophylline; VP, valproate.

[18, 16]. Antibody inhibition of alkoxyresorufin O-dealkylation by rat liver microsomes was studied using the lyophilised 50% ammonium sulphate precipitates of sera from immunised rabbits. Similar precipitates of sera from non-immunised rabbits served as pre-immune controls. The serum precipitates were washed once with standard phosphate-buffered saline (PBS, Oxoid), then lyophilised and stored at −80° until use, when a portion was dissolved in PBS at a concentration of approximately 20 mg protein/mL. Ammonium sulphate precipitated antibodies were used instead of immune serum because the fluorescence of the O-dealkylation metabolite, resorufin, was highly quenched by either whole serum or the supernatant remaining after ammonium sulphate precipitation, but not by the precipitated protein (data not shown). For the immunoinhibition experiments, freshly prepared antibody or pre-immune control solution was mixed in a fluorimeter cuvette with the correct amount of microsomal protein needed for the reaction, plus reaction buffer to a total volume of 0.1 mL, and the mixture stood for 20 min on ice with occasional swirling to allow the interaction between antibodies and P450, whereupon the remaining reaction buffer and substrate were added, followed by NADPH to start the reaction. The pre-immune serum protein preparation, at a concentration of 20 mg per mg microsomal protein, produced an effect on the microsomal O-dealkylation reactions ranging from 10% inhibition to 50% stimulation, depending on the combination of substrate and type of microsomes (data not shown). This was assumed to be a non-specific effect and to be common also to the antibody preparations: it was compensated for by maintaining the total concentration of antibody plus pre-immune protein constant at 20 mg per mg microsomal protein by mixing antibodies and pre-immune protein in varying proportions in the cuvettes.

HPLC assay for alkoxyresorufin O-dealkylation. An HPLC assay was developed to investigate whether other metabolites in addition to resorufin were formed. The microsomal reaction was carried out as described above, but scaled down for a total volume of 0.5 mL in an Eppendorf tube, with shaking at 37° and without addition of authentic resorufin at the end. After a suitable period 0.5 mL of ice-cold acetone was added with vortexing and the coagulated microsomal protein precipitated by centrifugation at 2000 rpm for 5 min in a bench centrifuge. The supernatant was filtered at 0.45 μm through a 13 mm PVDF Gelman syringe filter and a 100 μL aliquot was injected on to the HPLC for analysis. The HPLC used a 25 cm Spherisorb 5 μm amino column at room temperature with elution at 1 mL/min by a linear gradient of 40–100% aqueous acetone changing over 10 min. Resorufin and alkoxyresorufins were monitored using fluorescence detection at 530 nm (excitation) and 585 nm (emission) with peak area integration (Hewlet-Packard 3390A computing integrator). The retention time for resorufin was 3.2 min and for the substrates ethoxy-, pentoxy-, heptoxy-, octoxy- and benzyloxyresorufin were 4.9, 7.4, 8.4, 8.8 and 6.7 min, respectively. The relationship between peak area and amount injected per 100 μL was linear over the range 1–50 pmol for resorufin, 50–500 pmol for ethoxy-, pentoxy-, heptoxy- and benzyloxyresorufin and 50–800 pmol

for octoxyresorufin. For microsomal assays octoxyresorufin (1 μM) was added as an internal standard after stopping the reaction with acetone. The peak area ratio relative to octoxyresorufin was linearly related to the concentration in the reaction filtrate over the range 10 nM–0.5 μM for resorufin and 0.1–5 μM for the substrates.

RESULTS

HPLC analysis of metabolites

The HPLC method was highly sensitive, with a limit of sensitivity of approximately 0.001 pmol resorufin and 5 pmol ethoxyresorufin per 100 μL sample injected (i.e. the amount giving an HPLC compound peak 3× higher than background noise). When acetone was used to stop the reaction the recovery from incubation mixtures was between 94 ± 3% and 98 ± 2% (mean ± SD, N = 4) for 1 μM concentrations of authentic resorufin or ethoxy-, pentoxy-, hexoxy-, or benzyloxyresorufin, but when the reaction was stopped with 0.5 mL methanol (as used by Pohl and Fouts in a modification of our original fluorimetric assay [21]), the recovery of resorufin and ethoxyresorufin was only 66.7 ± 2% and 80 ± 6%, respectively. In order to check whether other metabolites in addition to resorufin were formed during AROD reactions, the HPLC assay was used to measure the metabolism of ethoxy-, pentoxy-, heptoxy-, and benzyloxyresorufin (1 μM) by liver microsomes from untreated, PB- and 3MC-treated rats (AROD activities of human liver microsomes were not similarly checked, however). Incubation times of 5 min were used, with an amount of microsomal protein (0.005–1.5 mg depending on the substrate and the induction type of the microsomes) which ensured a linear reaction over this period when measured by the direct fluorimetric method. Both resorufin and the substrate were quantified in order to calculate the stoichiometry of the reaction. None of the substrates was degraded when incubated for 5 min with boiled microsomes, while resorufin was unchanged after incubation for 10 min with NADPH plus liver microsomes from untreated, PB- or 3MC-treated rats. The rates of resorufin formation and the percentage conversion of substrate to resorufin are summarised in Table 2. The rates of resorufin formation measured by HPLC were very similar to those measured here and reported previously [3] using the direct fluorimetric assay. Resorufin constituted greater than 87% of the substrate metabolised and was the only metabolite peak visible on HPLC in all but two cases. When benzyloxyresorufin was metabolised by microsomes from untreated rats resorufin accounted for only 81.3% of substrate metabolised, although there were no additional metabolite peaks observed by HPLC, whilst when pentoxyresorufin was metabolised by 3MC-induced microsomes there were two small metabolite peaks additional to resorufin and only 77% of the substrate metabolised was accounted for as resorufin (Fig. 1). These two extra metabolites of pentoxyresorufin were not chemically identified. However, they may have retained the alkoxy side chain since they fluoresced more strongly at the optimum wavelengths for pentoxyresorufin (ex = 460 nm, em = 570 nm) than for resorufin (ex = 530 nm, em = 585 nm), whilst their retention times indicated them to be more polar than pentoxyresorufin. Alkoxyresorufin metabolism by human liver microsomes was not checked by HPLC analysis.

Specificities of purified rat P450 forms

The effect of altering the P450:NADPH-cyt. c reductase ratio was measured for CYP1A1, CYP1A2 and CYP2B1 with ethoxyresorufin, pentoxyresorufin and benzyloxyresorufin as substrates. In each case the ratio of 7.7 units of reductase per nmol P450 used gave a higher activity than ratios between 10-fold higher or 10-fold lower (data not shown). The

Table 2. Percentage and rate of conversion of alkoxyresorufins to resorufin during metabolism by rat liver microsomes

Substrate	Type of microsomes	Percentage of substrate converted to resorufin	Rate of resorufin formation (nmol/min/mg protein)
Ethoxyresorufin	UT	98.6 ± 2.1	0.472 ± 0.151
	PB	88.2 ± 3.2	3.041 ± 2.141
	3MC	90.3 ± 5.7	30.191 ± 1.615
Pentoxyresorufin	UT	87.7 ± 1.5	0.016 ± 0.013
	PB	91.4 ± 6.5	4.610 ± 3.521
	3MC	76.6 ± 8.9	0.111 ± 0.057
Heptoxyresorufin	UT	92.8 ± 2.7	0.011 ± 0.001
	PB	92.7 ± 2.9	0.018 ± 0.002
	3MC	86.9 ± 1.7	0.006 ± 0.001
Benzyloxyresorufin	UT	81.3 ± 6.4	0.137 ± 0.015
	PB	93.9 ± 4.1	16.370 ± 3.246
	3MC	89.6 ± 8.1	1.716 ± 0.262

Values are means ± SD for triplicates. Resorufin and substrates were measured by HPLC, using peak area ratios against the internal standard, octoxyresorufin, as described in the Materials and Methods. UT, PB and 3MC, liver microsomes from untreated, PB-treated and 3MC-treated rats respectively.

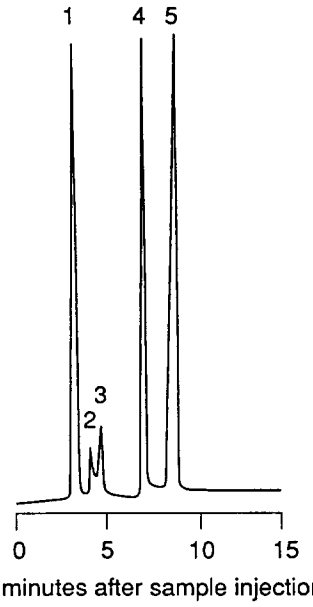

Fig. 1. High-performance liquid chromatogram of metabolites formed during metabolism of pentoxyresorufin by 3-methylcholanthrene-induced rat liver microsomes. Pentoxyresorufin (1 μM) was incubated for 5 min with microsomes (0.6 mg protein) and analysed by HPLC with fluorescence detection as described in Materials and Methods. Peak identities are: 1, resorufin; 2 and 3, unidentified; 4, pentoxyresorufin; 5, octoxyresorufin (internal standard). The abscissa scale is the time after sample injection in minutes.

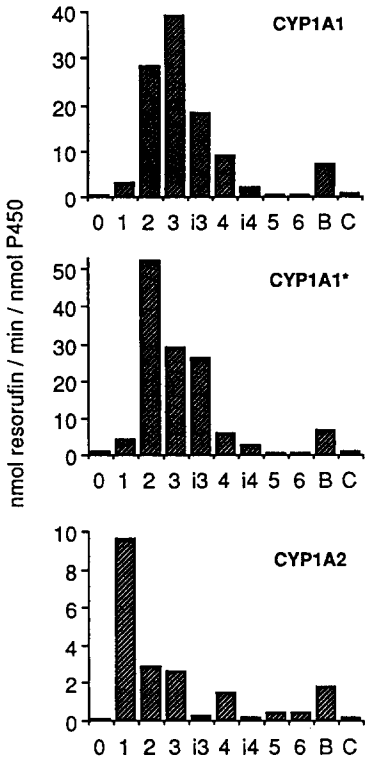

Fig. 2. O-Dealkylation of a homologous series of alkoxyresorufins by purified rat P450 CYP1A forms. CYP1A1, CYP1A1* and CYP1A2 were individually incubated with individual alkoxyresorufin homologues and the resorufin formed was measured by direct fluorimetry as described in Materials and Methods. Substrate abbreviations are: 0, phenoxazone; 1, methoxyresorufin; 2, ethoxyresorufin; 3, *n*-propoxyresorufin; i3, *iso*-propoxyresorufin; 4, *n*-butoxyresorufin; i4, iso-butoxyresorufin; 5, pentoxyresorufin; 6, hexoxyresorufin; B, benzyloxyresorufin; C, cyclohexoxyresorufin. Results are means of duplicate determinations, with replicates differing by less than 10% of the mean.

effect of adding purified rat liver cytochrome b5 to the reconstitution mixture was investigated for CYP1A1, CYP1A1*, CYP1A2 and CYP2C6 with ethoxyresorufin, for CYP2B1 and CYP2C6 with pentoxyresorufin and for CYP1A2, CYP2B1 and CYP2C6 with benzyloxyresorufin. P450:b5 ratios of 1:0.5, 1:1 and 1:5 had no significant effects on the reaction rates (data not shown).

The alkoxyresorufin substrate specificities of several purified forms of rat P450 in reconstituted monooxygenase systems are shown in Fig. 2 (CYP1A1, CYP1A1* and CYP1A2) and Fig. 3 (CYP2B1, CYP2B2 and CYP2C6). Note that several different ordinate scales have been used, in order to encompass the different turnover numbers of the forms. CYP1A1, CYP1A1* and CYP1A2 were selective for propoxyresorufin, ethoxyresorufin and methoxyresorufin, respectively (Fig. 2). The greatest differences in activity between rat CYP1A1 and CYP1A2 were in the metabolism of alkoxyresorufins with 2C and 3C chain lengths (the difference = 10, 15, 67 and 14-fold for EROD, PrROD and the O-dealkylations of iso-propoxy- and iso-butoxyresorufin, respectively). In contrast, the difference in activity between CYP1A1 and CYP1A2 was much less (<6-fold) for the non-substituted parent substrate, phenoxazone, and for alkoxyresorufins with either cyclic substituents or with *n*-alkyl substituents shorter than ethoxy or longer than propoxy. In a comparison of the CYP1A P450 activities where each was measured using its preferred substrate, CYP1A1 (measured using PrROD) was approximately 25% less active than CYP1A1* (measured using EROD) but approximately 300% more active than CYP1A2 (measured using MROD). CYP2B1 showed an overall selectivity for benzyloxy- and pentoxyresorufin (Fig. 3). CYP2B2 displayed very little activity at all, but was selective for benzyloxyresorufin. CYP2C6 was much less active than CYP2B1 or any of the CYP1A forms but showed distinct selectivities for benzyloxy-, propoxy- and butoxyresorufin. The P450s also differed in their comparative metabolism of *n*- and *iso*-alkoxyresorufins. CYP1A2 and CYP2C6 metabolised *n*-propoxy- and *n*-butoxyresorufin between 8- and 23-fold faster than the respective iso-homologues, whereas for CYP1A1* and CYP2B2 there were virtually no differences in activity between the *n*- and *iso*-compounds. CYP1A1 and CYP2B1 showed a negligible difference in activity between *n*- and

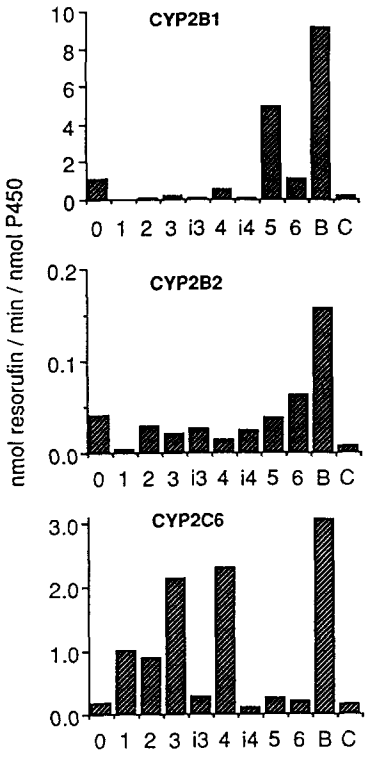

Fig. 3. O-Dealkylation of a homologous series of alkoxyresorufins by purified rat P450 CYP2 forms. CYP2B1, CYP2B2 and CYP2C6 were individually incubated with individual alkoxyresorufin homologues and the resorufin formed was measured by direct fluorimetry as described in Materials and Methods. Substrate abbreviations are as in Fig. 2. Results are means of duplicate determinations, with replicates differing by less than 10% of the mean.

iso-propoxyresorufin but a 4–5-fold difference between *n*- and *iso*-butoxyresorufin.

AROD specificities of P450 forms in rat liver microsomes

The major uses of the EROD and PROD reactions are as probes for CYP1A1 and CYP2B1, respectively, in hepatic microsomal suspensions, particularly in microsomes from experimentally induced animals in order to characterise and quantify the induction. Since the results with purified rat P450s showed that each alkoxyresorufin homologue could be metabolised by several alternative P450 forms, and since a measured activity could, in principle, be due equally well to either a small amount of a highly active P450 form or a large amount of a much less active form, it was important to establish which form or forms were actually responsible for the reactions in liver microsomes. This was studied by measuring the extent of inhibition of the microsomal reactions by antibodies to individual forms of P450. By this means the roles of CYP1A1, CYP1A2, CYP2B1, CYP2C6, CYP2C11 and CYP3A1/2 in catalysing MROD, EROD, PROD and BROD in control, PB-induced, 3MC-induced and ISF-induced rat liver microsomes were investigated (not every possible combination of reaction, microsome type and antibody was studied, however). Based on the evidence of immunoblots, the antibodies to CYP1A1 and CYP1A2 respectively cross-reacted weakly between the two purified CYP1A P450s and in 3MC- and ISF-induced rat liver microsomes, but showed no recognition of other P450 subfamilies (data not shown). There was, however, weak cross-reactivity across subfamilies among the antibodies to the CYP2B, CYP2C and CYP3A forms, while the anti-CYP2C6 and anti-CYP2C11 antibodies each recognised both purified CYP2C6 and CYP2C11 [18].

The inhibitions of EROD, PROD and BROD activities measured in liver microsomes prepared from untreated, PB-, 3MC- and ISF-treated rats by each of the various antibodies raised against different forms of P450 are shown in Figs 4–6. A near maximal inhibition for each combination of reaction, microsomes and antibody used was obtained using an antibody to microsomal protein ratio of 20:1. Examples where the maximum inhibition by individual antibodies summated to over 100% for the same reaction were probably due to antibodies cross-reacting with several P450 forms at the highest antibody concentrations used. The maximum antibody inhibitions observed are listed in Table 3 and can be summarised as follows (the inducer pretreatment of the rats is indicated as a prefix to the reaction). There was strong inhibition (>90%) by CYP1A1-Ab of 3MC-EROD and ISF-EROD, by CYP2B1-Ab of PB-EROD, PB-PROD, PB-BROD and ISF-BROD, and by CYP2C6-Ab of UT-EROD. There was moderate inhibition (40–90%) by CYP1A1-Ab of 3MC-PROD and 3MC-BROD, by CYP1A2-Ab of ISF-EROD and 3MC-PROD, by CYP2B1-Ab of UT-EROD, UT-BROD and ISF-BROD, by CYP2C6-Ab of PB-EROD, PB-PROD and PB-BROD, by CYP2C11-Ab of UT-EROD and UT-BROD, and by CYP3A1/2-Ab of UT-EROD, PB-PROD and PB-BROD. The remaining reaction–antibody combinations showed no significant inhibition (<40%).

AROD specificities of P450 forms in human liver microsomes

An increasingly frequent use of MROD, EROD and BROD reactions is to characterise the P450 activities of human liver microsomes. The roles of individual P450s in human hepatic microsomal MROD, EROD and BROD activities were investigated by antibody inhibition as for rats. The anti-rat CYP1A1, CYP1A2, CYP2C and CYP3A antibodies each specifically recognised the appropriate orthologous P450 subfamily in human liver microsomes on immunoblots, while the CYP2B1-Ab recognised CYP2A6, as described previously [16]. Both the CYP1A1-Ab and CYP1A2-Ab recognised a single band of molecular mass 53 kDa (human CYP1A2). Microsomal MROD and EROD activities in a single human liver (ID No. 10) were tested with a range of antibody concentrations up to 20 mg antibody per mg microsomal protein (Fig. 7). Both MROD and EROD were strongly inhibited by CYP1A1-Ab and CYP1A2-Ab, but not by CYP2B-

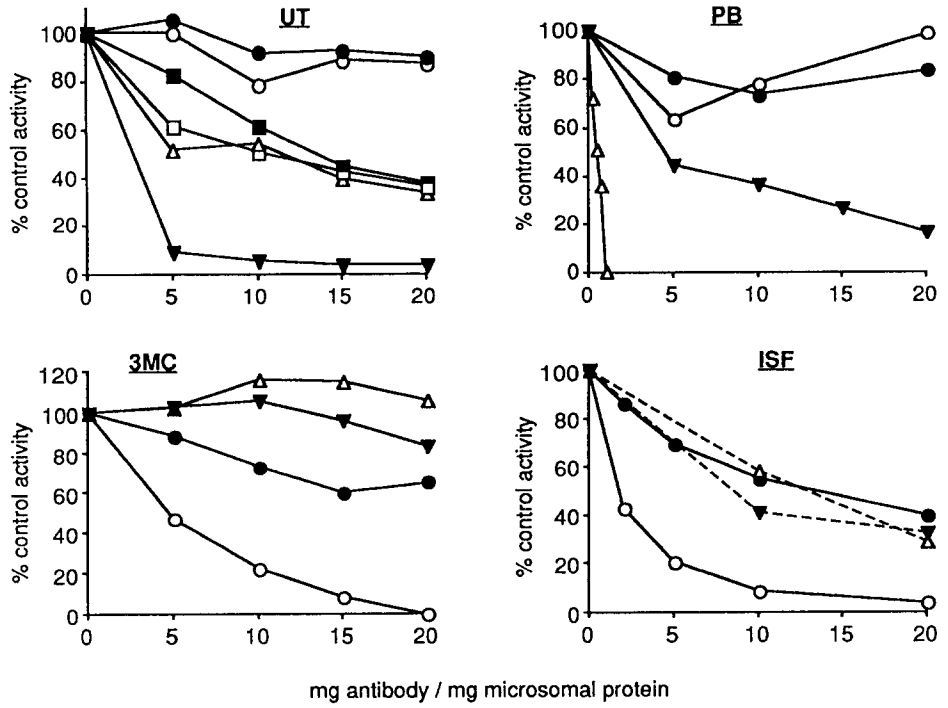

Fig. 4. Anti-P450 antibody inhibition of ethoxyresorufin O-deethylation and methoxyresorufin O-demethylation by rat liver microsomes. The reaction was carried out in the presence of varying concentrations of antibodies against individual P450 forms as described in Materials and Methods. Values are means of duplicates differing by less than 10% of the mean. 100% control activity is the activity in the presence of 20 mg pre-immune globulin per mg microsomal protein. *UT*, microsomes from untreated rats; *PB*, microsomes from phenobarbitone-treated rats; *3MC*, microsomes from 3-methylcholanthrene-treated rats; *ISF*, microsomes from isosafrole-treated rats. EROD was measured with UT, PB, 3MC and ISF microsomes (solid lines) and MROD was measured in addition with ISF microsomes (dashed lines), with antibodies as shown: EROD: —○— CYP1A1; —●— CYP1A2; —△— CYP2B1; —▲— CYP2C6; —□— CYP2C11; —■— CYP3A1/2; MROD: --▽-- CYP1A1; --▼-- CYP1A2.

Ab, CYP2C-Ab or CYP3A-Ab. Based on this result, microsomal MROD, EROD and BROD activities in a further nine human livers were tested with each of the antibodies at a single concentration (20 mg antibody per mg microsomal protein) (Table 4). In virtually every liver MROD and EROD activities were strongly inhibited by CYP1A1-Ab and/or CYP1A2-Ab, but not by CYP2B-Ab, CYP2C-Ab or CYP3A-Ab. However, the extent of anti-CYP1A antibody inhibition of MROD and/or EROD varied noticeably between livers, ranging from 60 to 98% inhibition (2–40% activity remaining). Also, there were individual examples of appreciable (i.e. at least 50%) inhibition of MROD and EROD by CYP3A-Ab (liver No. 6), CYP2B-Ab (livers No. 20 and 26) or CYP2C-Ab (livers No. 20 and 30). Antibody inhibition of BROD activity was more variable than the inhibition of MROD and EROD. The strongest BROD inhibition observed (92–98%) was caused by CYP3A-Ab, but this occurred in only three livers (Nos 6, 25 and 26), while in the other livers BROD inhibition by CYP3A-Ab was only moderate to weak (22–75% inhibition). BROD was appreciably (i.e. at least 50%) inhibited by CYP2B-Ab in five livers (Nos 6, 7, 19, 20 and 26) and by CYP1A2-Ab in two livers (Nos 26 and 31).

Furafylline inhibition of AROD activites in rat and human liver microsomes

Because the CYP1A1-Ab and CYP1A2-Ab cross-reacted to some extent between both CYP1A1 and CYP1A2, the question of which of these P450 forms were responsible for MROD and EROD activities was also addressed using the selective human CYP1A2 inhibitor, furafylline [22]. In four different human livers MROD activity was strongly (>90%) inhibited by 16 μM furafylline, while EROD was inhibited almost as strongly (>75% inhibition) (Fig. 8). At 2 μM concentration furafylline inhibited MROD and EROD by 45–80% in human liver microsomes. BROD activity was not inhibited by 16 μM furafylline in the single human liver tested (ID No. 26). Furafylline was less inhibitory in rat liver microsomes compared to human. Hepatic microsomal MROD activity was strongly (79–85%) inhibited by 200 μM furafylline in untreated and 3MC-treated rats, less strongly inhibited (62%) in ISF-treated rats and not appreciably inhibited (30%) in PB-treated rats (Fig. 9). Furafylline was less inhibitory at 80 μM than at 200 μM, but the lower concentration still caused marked inhibition (71%) of MROD in untreated rats. In contrast, EROD

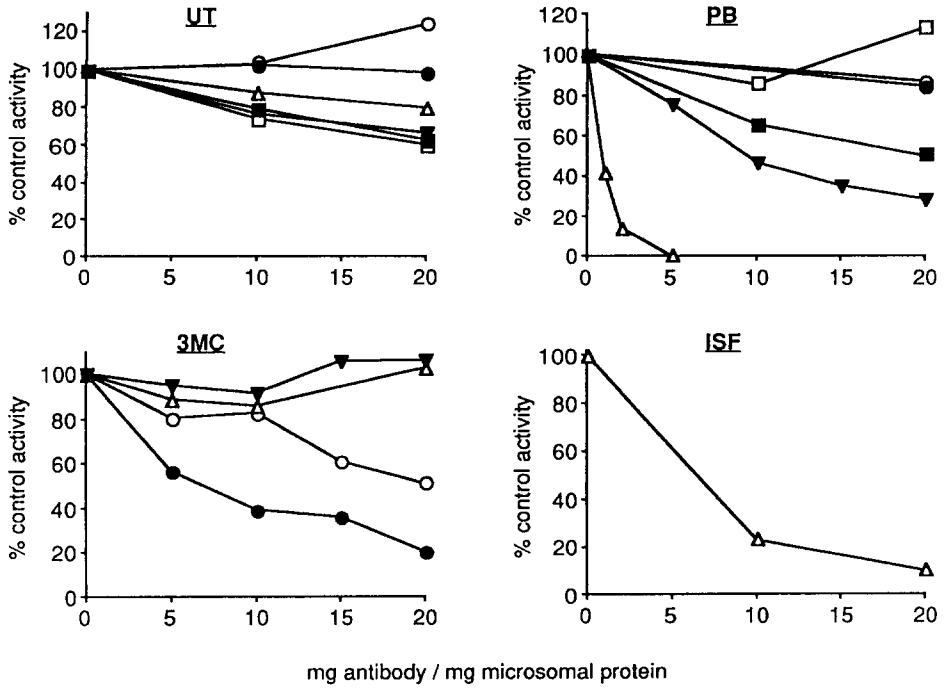

Fig. 5. Anti-P450 antibody inhibition of pentoxyresorufin O-dealkylation by rat liver microsomes. The reaction was carried out in the presence of varying concentrations of antibodies against individual P450 forms as described in Materials and Methods. Values are means of duplicates differing by less than 10% of the mean; 100% control activity is the activity in the presence of 20 mg pre-immune globulin per mg microsomal protein. *UT*, microsomes from untreated rats; *PB*, microsomes from phenobarbitone-treated rats; *3MC*, microsomes from 3-methylcholanthrene-treated rats; *ISF*, microsomes from isosafrole-treated rats. PROD was measured with antibodies added as shown: —○— CYP1A1; —●— CYP1A2; —△— CYP2B1; —▼— CYP2C6; —□— CYP2C11; —■— CYP3A1/2.

activity was not appreciably inhibited (23% or less) by 200 μM furafylline in rats, irrespective of treatment. BROD activity, tested only in ISF- and PB-treated rats, was similarly not inhibited by 200 μM furafylline.

DISCUSSION

In the 19 years since our original description of EROD as a direct fluorimetric assay for P450 monooxygenase activity and its selective induction by 3MC in rats [1], this and the homologous PB-inducible PROD reaction [4] have become virtually standard methods for measuring CYP1A1 and CYP2B1, respectively [3]. Confirmation of the high degree of selectivity of EROD for CYP1A1 and of PROD for CYP2B1 compared to other P450 forms, in rats and other species, has come from several laboratories using purified P450s [9, 23, 24], antibody inhibition [25] and expressed P450 cDNAs [14]. This study, by extending the specificity information to a wide range of homologous alkoxyresorufins, reinforces the remarkable differences in selectivity for these substrates between different rat P450s and confirms that ethoxyresorufin and pentoxyresorufin are the most discriminative of the homologues for assaying rat CYP1A1 and CYP2B1, respectively. Whilst there was no example in this study of an absolute specificity between one form of P450 and one alkoxyresorufin homologue, the rat P450 forms studied showed clearly differing patterns of substrate selectivity. Rat CYP1A1, CYP1A1* and CYP1A2 showed selectivity for propoxyresorufin, ethoxyresorufin and methoxyresorufin, respectively, CYP2B1 was selective for both pentoxyresorufin and benzyloxyresorufin and CYP2C6, while much less maximally active than these forms, was selective for benzyloxy-, propoxy- and butoxyresorufin. CYP1A1*, purified from 3MC-induced rats, was separated from CYP1A1 by column chromatography but has not been characterised other than having been shown to have the same N-terminal amino acid sequence and molecular weight on SDS–PAGE as CYP1A1 and to be recognised by polyclonal antibodies to CYP1A1 (data not shown). It is not known whether CYP1A1* is a distinct form of P450. Although only two forms (CYP1A1 and CYP1A2) are currently recognised in the rat CYP1A subfamily, a second form of human CYP1A1 has been discovered with an amino acid substitution that may affect its catalytic activity [26].

MROD, EROD, PROD and BROD activities are used in many laboratories as selective probes for CYP1A1, CYP1A2 and CYP2B1. In order for the absolute value of a single activity to be discriminative in this fashion, the reaction should be many times

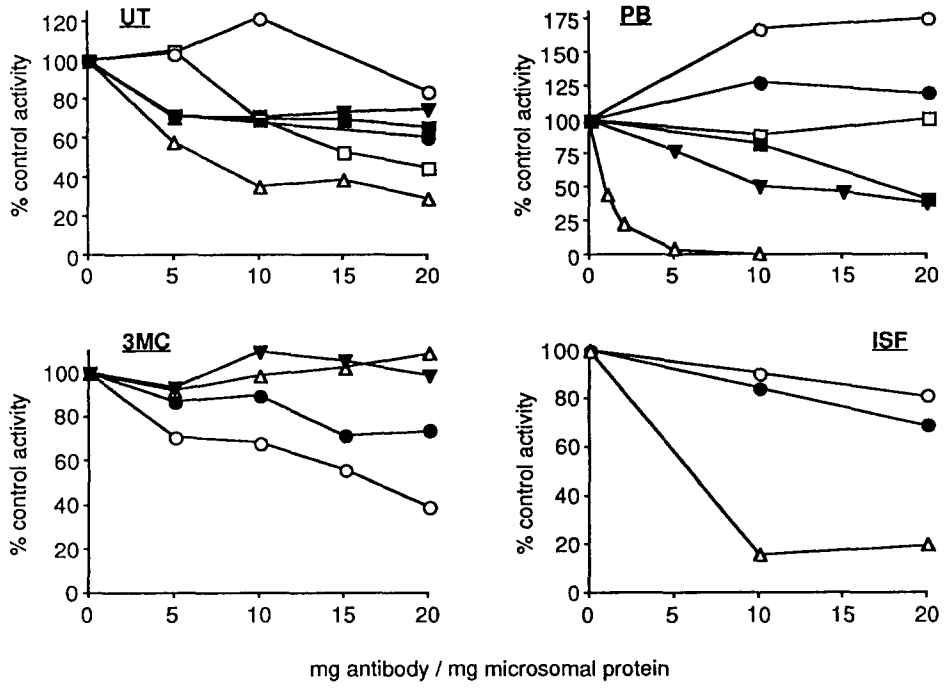

Fig. 6. Anti-P450 antibody inhibition of benzyloxyresorufin O-dealkylation by rat liver microsomes. The reaction was carried out in the presence of varying concentrations of antibodies against individual P450 forms as described in Materials and Methods. Values are means of duplicates differing by less than 10% of the mean; 100% control activity is the activity in the presence of 20 mg pre-immune globulin per mg microsomal protein. *UT*, microsomes from untreated rats; *PB*, microsomes from phenobarbitone-treated rats; *3MC*, microsomes from 3-methylcholanthrene-treated rats; *ISF*, microsomes from isosafrole-treated rats. BROD was measured with antibodies added as shown: —○— CYP1A1; —●— CYP1A2; —△— CYP2B1; —▼— CYP2C6; —□— CYP2C11; —■— CYP3A1/2.

Table 3. Summary of antibody inhibition of rat liver microsomal AROD activities

	Type of microsomes											
Antibody	UT	PB	MC	ISF	UT	PB	MC	ISF	UT	PB	MC	ISF
	EROD				*PROD*				*BROD*			
CYP1A1	n	n	s	s	n	n	m	—	n	n	m	w
CYP1A2	n	n	w	m	n	n	m	—	w	n	w	w
CYP2B1	m	s	n	—	n	s	n	s	m	s	n	m
CYP2C6	s	m	n	—	w	m	n	—	w	m	n	—
CYP2C11	m	—	—	—	w	n	—	—	m	n	—	—
CYP3A1/2	m	—	—	—	w	m	—	—	w	m	—	—

UT, liver microsomes from untreated rats. PB, MC and ISF, liver microsomes from PB-, MC- and ISF-treated rats respectively. n, w, m and s, no inhibition (<20%), weak inhibition (20–40%), moderate inhibition (40–90%) and strong inhibition (>90%), respectively, of EROD, PROD or BROD by an antibody (the maximum inhibition observed at up to 20 mg Ab per mg microsomal protein).— This particular combination of reaction, microsomes and Ab was not tested.

more active with one form of P450 than with any other form. According to this criterion, EROD was not effective in discriminating between CYP1A1 and CYP1A1* but did successfully discriminate CYP1A1 from CYP1A2, CYP2B1 and CYP2C6. (The EROD activities of CYP1A2, CYP2B1 and CYP2C6 were only 10%, 0.2% and 3% of CYP1A1 EROD activity, respectively.) PrROD showed a similar discrimination capability to EROD. By the same criterion PROD discriminated CYP2B1 from the other P450 forms measured (the PROD activities of which were <11% of CYP2B1). However, MROD, which has been suggested as a selective probe for rat CYP1A2 [6, 7], did not effectively discriminate

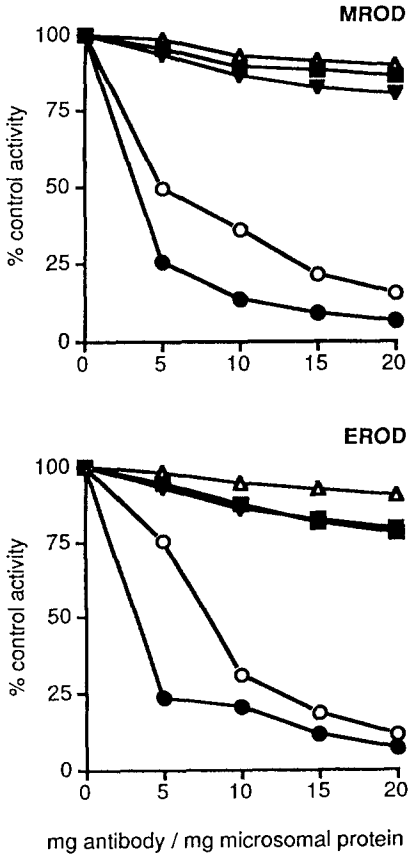

Fig. 7. Anti-P450 antibody inhibition of methoxyresorufin and ethoxyresorufin O-dealkylations by human liver microsomes. The reactions were carried out using liver microsomes from individual ID No. 10 in the presence of varying concentrations of antibodies against individual P450 forms as described in Materials and Methods. Values are means of duplicates differing by less than 10% of the mean; 100% control activity is the activity in the presence of 20 mg pre-immune globulin per mg microsomal protein. MROD, methoxyresorufin O-demethylation. EROD, ethoxyresorufin O-decthylation. Antibodies against individual rat P450 forms were added as shown: —○— CYP1A1; —●— CYP1A2; —△— CYP2B1; —▼— CYP2C6; —□— CYP2C11; —■— CYP3A1/2.

between CYP1A2 and either CYP1A1 or CYP1A1* (since CYP1A2 MROD activity was only 2–3-fold higher than CYP1A1 and CYP1A1* MROD activities), although furafylline inhibition pointed to a different conclusion in liver microsomes, as discussed below. Similarly, BROD failed adequately to discriminate between CYP1A1, CYP1A1*, CYP1A2, CYP2B1 and CYP2C6 (the largest difference in BROD activities was only 5-fold, between CYP2B1 and CYP1A2).

This study has highlighted the superiority of using the ratios between two AROD activities as a means of discriminating between different P450s compared to the use of single absolute AROD values. Thus, the best discriminator between CYP1A2 and the other P450 forms tested here was the *ratio* between MROD and PrROD activities (MROD : PrROD = 3.7 for CYP1A2 but only 0.1–0.5 for CYP1A1, CYP1A1* and CYP2B1, giving a 25–46-fold difference between CYP1A2 and CYP1A1 or CYP1A1*, compared to the 2–3-fold difference in MROD activities per se). Likewise, comparing purified CYP2B1 with the other P450 forms, whereas the difference was only 9–20-fold for PROD activity per se, it was 350–9700-fold for the EROD : PROD ratio.

Because the molecular environment is different between purified and membrane-bound forms of P450, the specificities of purified P450 forms in reconstituted systems might not properly reflect the activities of the same forms in microsomes. Moreover, since in control liver microsomes inducible forms of P450 are present in only small amounts relative to constitutive forms [23], the question arises of whether, for example, the slight EROD activity of control rat liver microsomes is due to the very small amount of highly active CYP1A1 or to the much larger amount of weakly active constitutive CYP2C6.

Discussion here of the antibody inhibition results concentrates on examples where one antibody inhibits much more strongly than all the other antibodies, indicating that the P450 form recognised by the strongly inhibitory antibody plays a major role in the reaction. This avoids the problem, arising particularly in cases of moderate inhibition by several antibodies, of ambiguity due to possible antibody cross-reactivity. The antibody inhibition results confirm that EROD was catalysed in both 3MC-induced and ISF-induced rat liver microsomes mainly by the CYP1A subfamily, with an apparently greater role for CYP1A1 than CYP1A2 (there will be no distinction between CYP1A1 and CYP1A1* when discussing antibody inhibition, since the anti-CYP1A1 antibody reacted equally strongly with CYP1A1*). This pre-eminence of CYP1A1 over CYP1A2 for EROD in 3MC-induced microsomes, also reported by Kelley *et al.* [27] and confirmed here by a lack of effect of the selective CYP1A2 inhibitor, furafylline [22], accords with the higher concentration of CYP1A1 than CYP1A2 in our 3MC-induced rat liver microsomes (immunoblotting data, not shown) and the greater intrinsic EROD activity of purified CYP1A1 compared to CYP1A2. However, even considering this difference in intrinsic EROD activities, the indication of a predominant role for CYP1A1 in ISF-induced microsomal EROD (confirmed by a lack of furafylline inhibition) might seem unexpected, since ISF induces much less CYP1A1 than CYP1A2 [23, 27] (also our immunoblotting data, not shown). In the case of MROD with ISF-induced microsomes, CYP1A1 and CYP1A2 appeared to be equally responsible for the reaction, which again might appear surprising in view of the preponderance of CYP1A2 after ISF induction and the higher intrinsic MROD activity of CYP1A2 compared to CYP1A1. A likely explanation for the less-than expected role of CYP1A2 in ISF-treated rats is partial inhibition of the induced CYP1A2 by an ISF metabolite adduct [28]. In contrast, extensive furafylline inhibition of MROD in 3MC-induced microsomes, with less inhibition in

Table 4. Antibody inhibition of human liver microsomal AROD activities

	P450 form or subfamily against which the antibody was raised				
Liver ID	CYP1A1	CYP1A2	CYP2B	CYP2C	CYP3A
	% Control activity remaining in the presence of antibody*				
			MROD activity		
6	39	15	107	68	46
7	19	7	63	101	72
10	15	6	90	80	86
13	4	19	nm	nm	96
19	23	14	96	62	100
20	15	6	86	96	108
25	17	9	92	80	85
26	2	2	104	120	100
30	13	9	80	50	91
31	12	16	93	92	98
			EROD activity		
6	16	26	81	88	95
7	19	2	67	86	100
10	11	7	90	79	78
13	11	13	nm	nm	107
19	19	2	88	92	72
20	40	7	50	30	71
25	2	27	87	133	100
26	2	2	53	59	70
30	17	7	82	68	71
31	30	7	89	73	83
			BROD activity		
6	82	64	50	59	2
7	85	56	39	115	25
10	nm	nm	nm	nm	nm
13	nm	nm	nm	nm	nm
19	56	52	47	63	43
20	55	68	45	88	41
25	102	62	60	103	8
26	61	22	39	78	8
30	70	73	97	90	73
31	78	27	53	92	78

* The antibody concentrations were all 20 mg antibody per mg microsomal protein. The values (means of duplicate determinations) are percentages of the activity which had been observed in the presence of 20 mg pre-immune serum globulin per mg microsomal protein. The pre-immune preparation itself in each case caused <10% inhibition or stimulation of the reaction. nm, not measured.

ISF-induced microsomes, indicated that CYP1A2 played the major role in catalysing 3MC-induced MROD and confirmed a relatively lesser contribution by CYP1A2 to ISF-induced MROD. It has similarly been deduced, as a result of antibody inhibition, that CYP1A2 plays a major role in MROD but not EROD in polychlorinated biphenyl-induced F344 rats [7]. Our previous report that the selective CYP1A inhibitor, ANF [29, 30], strongly inhibits EROD in 3MC-induced but not control rat liver microsomes [31], was an early indication that constitutive EROD is catalysed by P450 forms other than CYP1A. We have now confirmed this, showing by antibody inhibition and a lack of furafylline inhibition that EROD in control microsomes appeared to be catalysed mainly by CYP2C6. This largely corroborates a previous report that CYP2C11 is mainly responsible for constitutive EROD (the antibody used in that study cross-reacted with CYP2C6) [25] but contradicts an alternative suggestion that CYP1A2 is the main enzyme involved in untreated rats [27]. Extensive furafylline inhibition indicated, however, that MROD activity in liver microsomes of untreated rats was due mainly to constitutive CYP1A2. This difference in the EROD and MROD roles of CYP1A2 and CYP2C6 might be explained by a consideration of their intrinsic activities and their constitutive microsomal levels: in our study purified CYP1A2 was more active than CYP2C6 in both reactions but the difference was larger for MROD (10-fold) than for EROD (3-fold), while CYP2C6 is present in more than 12-fold excess over CYP1A2 in untreated adult male Sprague–Dawley rats and CYP2C6 and CYP2C11 combined are in more than 50-fold excess [23]. In PB-induced microsomes EROD activity appeared, surprisingly, to be due mainly to CYP2B1, which contradicts a previous suggestion that PB-induced CYP2C6 is mainly responsible [25]. A lack of furafylline inhibition also indicated that neither EROD nor

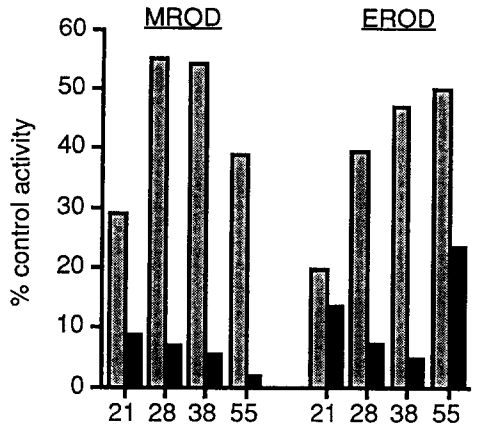

Fig. 8. Furafylline inhibition of methoxyresorufin and ethoxyresorufin O-dealkylations by human liver microsomes. Methoxyresorufin O-demethylation (*MROD*) and ethoxyresorufin O-deethylation (*EROD*) reactions were carried out using liver microsomes from individuals ID. Nos 21, 28, 38 and 55 in the presence of either 2 μM ▨ or 16 μM ■ furafylline as described in Materials and Methods. Values are means of duplicates differing by less than 10% of the mean; 100% control activity is the activity in the absence of furafylline.

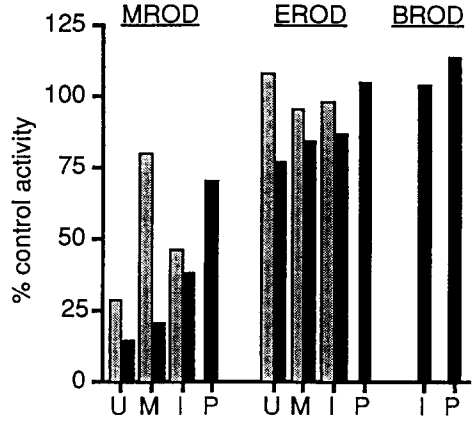

Fig. 9. Furafylline inhibition of methoxyresorufin, ethoxyresorufin and benzyloxyresorufin O-dealkylations by rat liver microsomes. Methoxyresorufin O-demethylation (*MROD*), ethoxyresorufin O-deethylation (*EROD*) and benzyloxyresorufin O-dealkylation (*BROD*) reactions were carried out using liver microsomes from untreated (U), 3-methylcholanthrene-induced (M), isosafrole-induced (I) or phenobarbitone-induced (P) rats in the presence of either 80 μM ▨ or 200 μM ■ furafylline as described in Materials and Methods. Values are means of duplicates differing by less than 10% of the mean; 100% control activity is the activity in the absence of furafylline.

MROD in PB-induced microsmes were catalysed mainly by CYP1A2. According to these results, therefore, EROD was not an effective measure of CYP1A1 or CYP1A2 in control or PB-induced rat liver microsomes, although it is unfortunately used as such by many researchers. MROD, however, was an indicator of CYP1A2 activity in control and 3MC-induced rat liver microsomes, but not in PB-induced microsomes.

With PROD, although the antibody inhibition results confirmed that it is an excellent probe for CYP2B1 in PB-induced rat liver microsomes and probably also in ISF-induced microsomes (ISF induces CYP2B1 in addition to CYP1A1 and CYP1A2 [23, 27]), no single one of the P450 forms tested here appeared to be mainly responsible for PROD in control microsomes. 3MC marginally induces microsomal PROD (9-fold compared to 280-fold by PB [3]) and the 3MC-induced PROD activity appeared, on the evidence of antibody inhibition, to be catalysed mainly by CYP1A1 and CYP1A2. The degree of inhibition of 3MC-induced PROD by polyclonal CYP1A1-Ab (49%) was almost exactly the same as reported by Nakajima *et al.* for a monoclonal CYP1A1-Ab [25], but they did not test for CYP1A2 involvement.

According to the antibody inhibition data, BROD appeared to be a measure of several P450 forms in liver microsomes of untreated rats, although principally CYP2C11 and CYP2B1 (although since there is very little CYP2B1 in control microsomes [23], this result may include an element of CYP2B1-Ab cross-reaction with a constitutive P450). In PB-induced and ISF-induced microsomes BROD was mainly due to CYP2B1, whereas in 3MC-induced microsomes the main contributor to BROD was the CYP1A subfamily. These data accord well with the intrinsic BROD activities of CYP1A1 and CYP2B1 and their preponderance in 3MC- and PB-induced microsomes, respectively. In various reports BROD has been considered to be an indicator mainly of either CYP2B [32, 7] or CYP3A [9, 33] in rats. This study shows that several rat P450s exhibit BROD activity and that the form(s) primarily responsible for BROD in liver microsomes vary according to how the rats have been induced.

In human liver microsomes, in contrast to rat, the results of antibody and furafylline inhibition indicated that both EROD and MROD were metabolised mainly by CYP1A2, although there was an interindividual variation in the extent of CYP1A2 involvement. This result is in accord with findings that human liver expresses CYP1A2 but not (with rare exceptions) CYP1A1 [34] and that expressed human CYP1A2 cDNA has many-fold higher MROD and EROD activity than other expressed human P450 cDNAs [14]. It corroborates and strengthens previous reports based on antibody inhibition that CYP1A2 catalyses EROD in adult human liver microsomes [35, 36] and that EROD and MROD correlate with the immunoquantified CYP1A2 concentration [15, 16]. In two livers in this study, however, there was also antibody inhibition evidence for a significant involvement of either CYP2C or CYP3A in human hepatic MROD or EROD activity. On the basis of antibody inhibition BROD activity in human liver microsomes appeared to be catalysed mainly by CYP3A in three livers, but by a concerted effort of CYP3A, CYP2A and CYP1A in other livers, with considerable

interindividual variation in the extents of involvement of the different P450s. This result is similar to our finding that BROD can be catalysed by several P450 forms in rats and is in accordance with reports that several different expressed human P450 cDNAs show similar levels of BROD activity [14, 37].

These results highlight the link between substrate structure and P450 specificity and exemplify, with CYP1A1*, CYP1A1 and CYP1A2, the remarkable differences in substrate selectivity that can occur between structurally highly related P450 forms. With the CYP1A P450s, as with 3MC-induced rat liver microsomes [3], the longer side-chain alkoxyresorufins and cyclohexoxyresorufin were O-dealkylated more slowly than the short-chain homologues. This effect of chain length is fairly common amongst P450 substrates [38] and could have been due to O-dealkylation being superseded by alicyclic ring hydroxylation with cyclohexoxyresorufin or by side-chain hydroxylation in the longer-chain alkoxyresorufins, as happens with alkoxynaphthalenes [39]. However, HPLC analysis showed that, with one exception (PROD in 3MC-induced rats), O-dealkylation to resorufin was the sole route of metabolism for both short- and long-chain alkoxyresorufins by uninduced or induced rat liver microsomes. The varying alkoxyresorufin substrate specificities of the different P450 forms cannot be explained by simple concepts of either alkoxyresorufin lipid solubility (which increases with increasing chain length [4]) or alkoxy side-chain bulk. The spatial fit of an alkoxyresorufin in the active site of a P450 is likely to be a major influence in substrate specificity, in which case large differences in reaction rates might be expected between *n*-alkoxyresorufins and *iso*-alkoxyresorufins. Interestingly, such isomer effects were much more pronounced in CYP1A2 and CYP2C6 than in other P450s, possibly indicating that substrate spatial fit is more critical for some P450 forms than others. It is also interesting that with every P450 form tested benzyloxyresorufin was a much more active substrate than cyclohexoxyresorufin. This might be further evidence for the importance of spatial fit, comparing the planar benzyl ring with the non-planar cyclohexyl ring, but the difference in "leaving group" electronic attributes between the two types of ring might also be important, since it has long been known that benzyl substituents are highly active leaving groups in the O-dealkylation of alkoxynitrophenyls [38].

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